

REVIEW

Association of chromosome damage detected as micronuclei with hematological diseases and micronutrient status

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Received on June 21, 2010; revised on September 7, 2010; accepted on September 8, 2010

Epidemiological studies reveal strong association between micronutrient deficiencies and development of cancer. Since chromosome breaks and abnormal chromosome segregation, identified as micronuclei (MN), are central to malignant transformation, the influence of micronutrient status upon MN frequency has been the subject of intense research. Motivating this effort is the idea that marginal micronutrient deficiencies lead to allocation of scarce cellular resources towards immediate survival at the expense of maintaining genomic integrity, placing the individual at greater risk for degenerative diseases and cancer in old age. The challenge in identifying an association between individual micronutrients and MN frequency stems from the complexity of human diet, simultaneous presence of multiple micronutrient deficiencies, variable genetic susceptibility and methodological difficulties. A unique model for studying MN in humans is provided by a group of haematological diseases, the chronic haemolytic anaemias associated with high reticulocyte count and absence of splenic function. These disorders may prove valuable for assessing the influence of micronutrient status once the effect of abnormal erythropoiesis on MN formation is adequately understood. Eventually, large population-based studies that can account for the baseline variability in MN frequency, lifestyle and genetic factors may be needed to uncover the DNA-damaging effect of poor diet. Understanding the link between micronutrient status and MN frequency will contribute towards determining optimal micronutrient intake to preserve long-term health.

Introduction

An increase in the frequency of micronuclei (MN), which reflects occurrence of chromosome breaks and abnormal chromosome segregation, is associated with future risk of cancer in humans (1). The measurement of MN has been utilised to explore the effect of genotoxic agents in populations and has also become a valuable tool to study the link between nutrition and DNA damage (2).

Micronutrients are a set of ~40 substances, the vitamins, essential minerals and other compounds required in small

amounts for normal metabolism, that are essential for human health (3–5). The optimum micronutrient intake for preserving long-term health is difficult to ascertain and may be higher than the current dietary recommendations (3). The magnitude of the worldwide problem of micronutrient malnutrition is immense even by the current standards (6). Inadequate micronutrient intakes are widespread in poor countries, and in the affluent nations, large segments of society, especially the poor, children, adolescents, the obese and the elderly do not meet the dietary reference intakes (7,8). Over half of the US population consumes inadequate magnesium, and large segments of the population are extremely low in vitamin D, omega-3 fatty acids, potassium, calcium, vitamin C, vitamin E and vitamin K (9). Given the scope of the challenge, the demonstration of a link between micronutrient malnutrition and DNA damage through the measurement of MN frequency will aid in stepping up implementation of public health strategies to reduce diseases of ageing and cancer. These issues have been discussed at length in a recent publication (10).

In this review, we will consider experimental evidence linking MN frequency in humans, as measured by the erythrocyte MN assay, to haematological diseases. The erythrocyte MN assay has been used less often in large human studies as opposed to the lymphocyte cytokinesis-block MN assay. The technical difficulty of using the erythrocyte MN assay partly derives from the active removal of the MN-containing reticulocytes from circulating blood by the spleen (11). Therefore, a group of haematological diseases, chiefly the chronic haemolytic anaemias associated with the absence of splenic function and high reticulocyte count, forms an attractive and convenient model for the application of erythrocyte MN assay in peripheral blood samples. We will examine the usefulness and limitations of haematological diseases to evaluate genotoxicity in humans.

MN and micronutrient status

DNA damage develops rapidly during micronutrient deficiencies (12,13), but the consequences of this damage, cancer and degenerative diseases, are delayed in onset (3). This temporal dissociation can be explained by redistribution of limited nutritional resources through evolutionary adaptation of biochemical pathways (9,10,14). As the scarcity of a micronutrient increases, a triage mechanism for allocating scarce micronutrients is activated that favours short-term survival at the expense of long-term health, in part through an adjustment of the binding affinity of each coenzyme for its required micronutrient (9). An important corollary of the 'triage theory' (9,14) is that the degenerative diseases accompanying ageing might be delayed by an inexpensive micronutrient intervention (14). There is little societal concern because no overt pathologies have been associated with marginal to moderate levels of deficiency. But the pathology is insidious in the short term, and the chronic degenerative disease of the future is

presaged by the DNA damage observed with micronutrient deficiencies.

Substantial experimental evidence supports the critical role played by micronutrients in the preservation of genomic integrity (15,16). We have shown the deficiency of several micronutrients (iron, magnesium, zinc and vitamins B₆, C, folic acid and biotin) results in increased DNA damage in primary human cells in culture or in rodents (3). Other studies have documented DNA damage in humans who are moderately deficient in iron, zinc, folate and B₁₂ or choline and in rodents or human cell cultures for mostly severe deficiencies in selenium, copper, calcium, niacin and choline (9,10,17). The depletion of zinc, which is a cofactor for several DNA repair pathways, increases single-strand breaks that return to normal upon zinc repletion (18). The vitamin K-dependent transforming growth factor beta-inducible protein (Tgfb β) is critical to integrity of the mitotic spindle, and mouse-knockout model demonstrates increased chromosomal aberrations and spontaneous cancer (14,19). Human intervention trials with micronutrients report a decrease in DNA damage (20), though more studies are needed to reach a definitive conclusion.

Micronutrient status is associated with cancer in epidemiological studies. High serum copper, high serum iron, low serum magnesium and concomitance of low serum zinc with high serum copper or low serum magnesium contribute to an increased mortality from cancer in middle-aged men (21,22). Low vitamin B₆ intake correlates with the risk for colorectal cancer, and the risk is further increased by alcohol intake (23). Meta-analysis of nine prospective studies estimated that risk of colorectal cancer decreased by 49% for every 100 pmol/ml increase in blood pyridoxal 5'-phosphate (24). Low magnesium consumption also increases the risk of colorectal cancer (25).

Assay methodologies used for evaluating the link between MN frequencies and micronutrient status are the lymphocyte cytokinesis-block assay, the erythrocyte MN assay (and the related reticulocyte MN assay) and MN formation in exfoliated cells (13,26–28). Studies using these techniques show that dietary adequacy of micronutrients affects the frequency of spontaneous MN in humans. Owing to the complexity of human diet, the relationship between individual micronutrients and MN frequency can be difficult to interpret, unlike *in vitro* experiments or animal models. The association of folate (and B₁₂) status with MN formation is clearly an exception to this observation (Table I).

Folate status and MN frequency

The folate coenzymes are required for *de novo* synthesis of nucleotides used for DNA replication and for DNA methylation. Hence, folate deficiency has a genome destabilising effect (33). The profound effects of folate deficiency on DNA integrity and MN formation arise from decreased N⁵, N¹⁰-methylene tetrahydrofolate availability, which reduces synthesis of thymidylate (dTMP) (33). The result is an increase in the cellular dUMP/dTMP ratio and DNA polymerase-mediated deoxyuridine triphosphate misincorporation into DNA (34). Excision of uracil from DNA generates single-strand breaks that could result in a double-strand break if two opposing nicks are formed. These mechanistic studies established that the deleterious effects of folate deficiency on DNA integrity develop prior to the appearance of overt haematological manifestations.

The initial visible manifestation of severe folate deficiency affects the haematopoietic system (35) in the form of abnormal

Table I. Studies on micronutrient status and MN frequency in RBCs

Reference	Population	Cell ^a	Association with MN Frequency
Observational			
Smith <i>et al.</i> (29)	Normal	E	Increase with calcium supplementation ($P = 0.05$) Decrease with supplements of C, E or A ($0 = 0.02$)
Schreinemachers and Everson (30)	Normal	E	Inverse relation with serum folate ($P = 0.004$)
Tucker <i>et al.</i> (31)	Normal and folate-deficient	E	Increase with fried beef consumption in folate-deficient group ($P < 0.05$)
Blount <i>et al.</i> (32)	Normal and folate-deficient	E	Increase in folate deficiency ($P < 0.05$)
MacGregor <i>et al.</i> (28)	Normal	E	Increase with low folate or B ₁₂ status ($P < 0.05$)
Abramsson-Zetterberg <i>et al.</i> (26)	Normal	R	Decrease with higher folate ($P < 0.05$)
Interventional			
Everson <i>et al.</i> (12)	Folate-deficient	E	Decreased with folate supplementation
Blount <i>et al.</i> (32)	Normal and folate-deficient	E	Decrease in folate-deficient with supplementation ($P < 0.05$)
Abramsson-Zetterberg <i>et al.</i> (26)	Normal	R	No change with folate supplementation

^aCell type, E, erythrocyte; R, reticulocyte.

nuclear maturation of precursors of blood cells in the bone marrow. Thus, the erythrocyte MN assay is particularly suited to the study of folate inadequacy. This was demonstrated in a splenectomised adult with mild folate deficiency, who had marked increased in MN erythrocytes compared to folate-replete individuals, and rapidly corrected to the normal value after treatment with folate (12). Subsequently, analysis of blood samples from >150 splenectomised subjects showed that individuals with high observed MN frequencies (>0.1 per 100 erythrocytes) were all deficient in either folate or B₁₂ (32). With the development of the flow cytometry-based reticulocyte MN assay, effect of folate status on MN frequency was also examined in non-splenectomised individuals (26). This study showed significant negative correlation between serum folate level and reticulocyte MN frequency in 99 subjects who all had serum folate levels within the normal range. Folate repletion reduces MN formation in deficient individuals, but supplementation has no effect on MN frequency when folate status is normal (26,36).

Despite the uniformity among various studies in identifying the strong link between folate status and MN frequency, there is wide individual variability of MN frequency with normal folate status (26). Consequently, apart from a state of extreme deficiency, MN frequency cannot be predicted from folate status with certainty. Whether this variability in MN is from inherited traits, influence of other micronutrients or the environment has not been determined.

Haematological diseases

Haematological diseases provide both an opportunity and a challenge for the evaluation of genotoxicity using the MN

assay (37,38). The flow cytometry-based erythrocyte MN assay is particularly suited to these diseases for two reasons. First, the reticulocyte count is 5- to 10-fold higher in haemolytic anaemias compared to normal (39) to compensate for early destruction of red blood cells (RBCs) (Figure 1a–c). This makes the enrichment procedures for reticulocytes from whole blood considerably more efficient. Second, splenectomy is common in several haematological conditions and sickle-cell anaemia (SCA), which affects millions of individuals, is associated with a lack of splenic function (39). In the absence of splenic filtration, micronucleated reticulocytes persist in the peripheral blood, mature as micronucleated erythrocytes and can be easily enumerated with flow cytometry (40). The lack of the need to enrich blood samples for reticulocytes simplifies the entire assay.

Micronutrient deficiencies in haemolytic anaemias

Chronic haemolytic anaemias, such as thalassaemia and SCA, have remarkably high prevalence of micronutrient deficiencies (41–43). Factors influencing nutritional requirements in these diseases are high basal metabolic rate from anaemia and increased erythropoiesis (44), iron overload (45), oxidative stress (46), hepatic and renal dysfunction (47) and

the long-term use of iron chelators (deferrioxamine or others). The dietary requirement for certain micronutrients is higher than the normal population, as shown by vitamin C deficiency in heavily iron-overloaded patients (45). Oxidative stress is present and becomes particularly apparent with the development of transfusional hemosiderosis (46). Consequently, plasma levels of the dietary antioxidants are depleted in the majority of patients. Deficiencies of zinc, copper and vitamin D are also widespread from dietary inadequacy or increased requirement (41).

Folate

The micronutrient of most concern in patients with haemolytic anaemias is folate (42,48,49). Haemolysis shortens the lifespan of the RBCs, and the replacement rate for RBCs by the bone marrow increases from around $\geq 1\text{--}5\%$ /day (50). Erythroid progenitors in the bone marrow increase in number and proliferate, and the tremendous need for DNA replication requires adequate folate and other cofactors to allow DNA biosynthesis to proceed without disruption. Patients with haemolytic anaemias who are deficient in folate develop abnormal nuclear maturation in erythroid precursors (megaloblastic changes), which responds to folate supplementation (48,51).

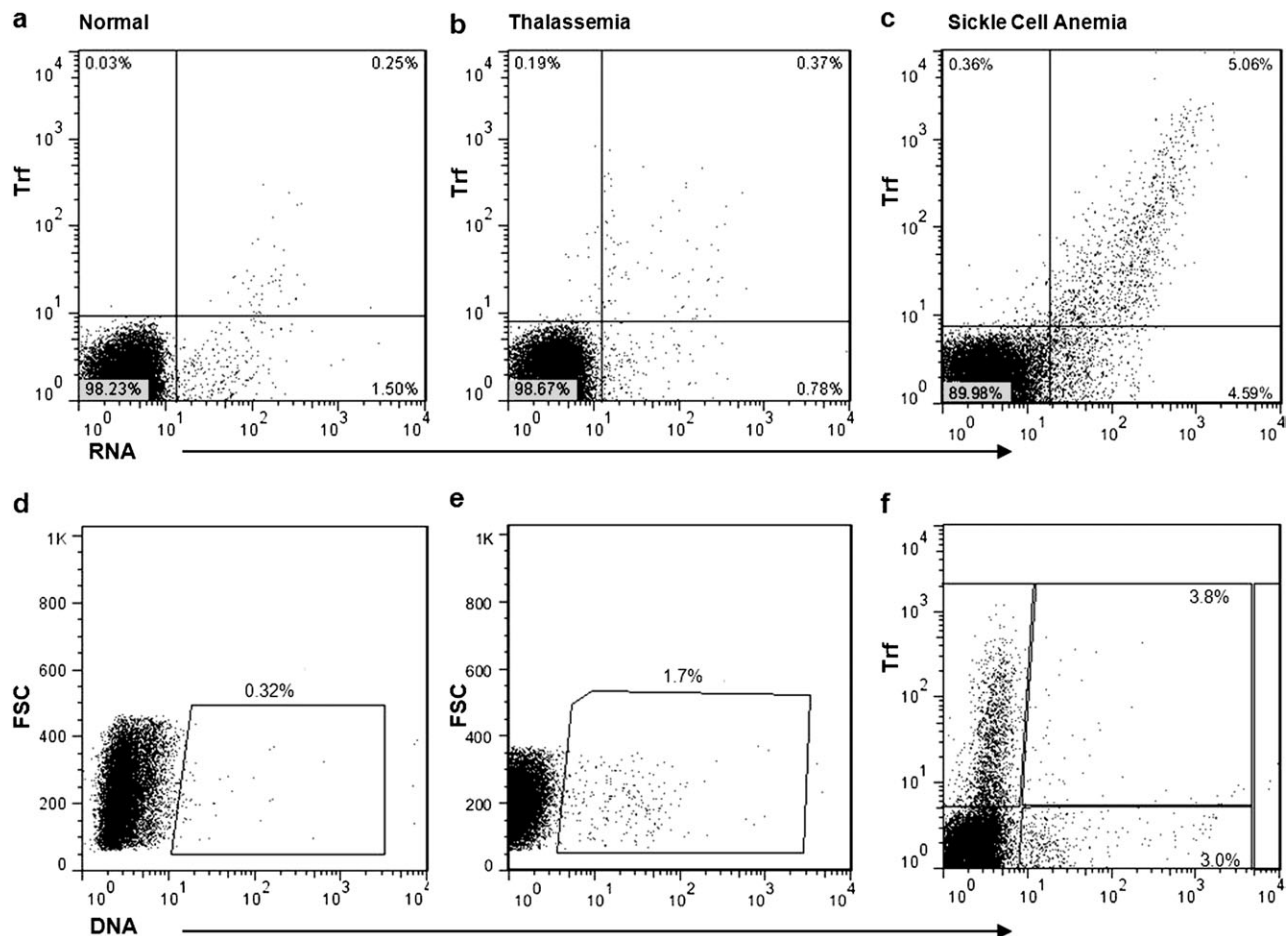


Fig. 1. (a–c) Reticulocyte maturation in peripheral blood: normal blood (a) has 0.5–2% reticulocytes (RNA+) and Trf is expressed on the least mature cells, which constitute ~20% of the reticulocyte population. In SCA (c), reticulocytes are 5–15% of the RBCs and more than half are Trf+, although expression of Trf decreases with maturation. Disordered reticulocyte maturation is observed in thalassaemia (b), where reticulocyte count is lower than anticipated for the degree of anemia, and no pattern in Trf expression is observed between less versus more mature reticulocytes. (d–f) MN RBCs (DNA+): as discussed in the text, reticulocyte enrichment is not necessary in SCA (f) to measure MN in mature erythrocytes (Trf–, DNA+) or immature reticulocytes (Trf+, DNA+). Reticulocyte enrichment increases observable MN events in normal (d) and thalassaemia (e). Increased MN–Trf+ RBCs are observed in thalassaemia with intact spleen (1.7%) and SCA with absent splenic function (3.8%) compared with normal blood (0.32%). In the absence of splenic filtration, MN can be observed in mature RBCs in SCA (3.0% in this example). FSC, forward scatterer.

Consequently, it is now customary to provide supplemental folate (usually as 1000 mg folic acid/ day) to patients with haemolytic anaemia (49). While this prevents overt megaloblastic changes, it is not certain whether adequacy is achieved at the level of all folate-dependent metabolic reactions. Marginal folate deficiency can increase chromosome damage, and this may contribute to elevated MN frequency in patients with thalassaemia and SCA (38,40).

Intrinsic erythroid defects

Micronucleated reticulocytes or erythrocytes in haemolytic anaemias may also arise from disruption of normal development of RBCs (erythropoiesis). There are increased number of immature RBC precursors in the bone marrow (52), reticulocytes are released early into the circulation, persist longer in blood before transforming into mature erythrocytes and demonstrate higher expression of transferrin receptor (Trf) (Figure 1a–c). These compensatory adaptations, even in the presence of intact splenic function, make the comparison of MN frequency between individuals with haemolytic anaemias and normal population difficult. In addition, the developing erythroid progenitor can be exposed to iron-catalysed free radicals (denatured globin–heme complexes) or abnormal suppression of apoptosis (erythropoietin effect), which can influence the rate of chromosome damage during maturation (53). The extreme outcome of this process is the early demise of erythroid progenitors, most evident in thalassaemia, where immature precursors vastly exceed the number of reticulocytes (ineffective erythropoiesis) (53). To a lesser extent, ineffective erythropoiesis also occurs in SCA, though not in hereditary spherocytosis (54).

Patients with thalassaemia and SCA have coexisting nutritional deficits (41) and iron-induced oxidative stress (46,55), both of which are expected to increase DNA damage. We have demonstrated elevated reticulocyte MN frequency in thalassaemia (38) (Figure 1) but the coexistence of ineffective erythropoiesis makes it difficult to extrapolate the increase in chromosome damage in RBCs to other organs. As discussed later, this suggests the need for examination of MN frequency in cells from other tissues in order to determine if the DNA damage is restricted to erythroid cells or is generalised.

Exposure to toxic drugs

Patients with SCA and thalassaemia are commonly treated with the antineoplastic drug hydroxyurea (HU, hydroxycarbamide) to induce the synthesis of foetal haemoglobin. Long-term treatment with HU lowers mortality in SCA and reduces need for blood transfusions in thalassaemia (56,57). The hydroxamic acid core of HU inhibits the iron-containing enzyme ribonucleotide reductase and by inducing death of cycling bone marrow cells, it alters the kinetics of erythropoiesis leading to greater synthesis of foetal haemoglobin (57). HU is directly mutagenic *in vitro* and prevents DNA repair, allowing deleterious lesions to accumulate with time (58). The prominent role of HU in SCA has led to careful examination of evidence of DNA damage with long-term use in patients. Overall, the *in vivo* mutagenic risk of HU appears to be low. Older adults with myeloproliferative disorders do not show increase in acquired somatic mutations (*HPRT* mutants or illegitimate VDJ-recombination events) when treated with HU (59). In children with SCA, however, longer HU exposure (30–40 months) was significantly more mutagenic compared to short-term exposure (59,60). The MN frequency measured by

the flow-cytometry based erythrocyte MN assay shows a consistent rise starting from the earliest time-point of 3 months from start of HU treatment (37). This difference in time course between the mutagenicity in peripheral blood mononuclear cells and DNA double-stranded breaks in erythrocytes suggests that aggravation of erythropoietic stress from HU (57) may be the mechanism of generation of MN-RBC, while defective DNA-repair results in accumulation of mutations after a sustained use (59).

The complex pathophysiology of haemolytic anaemias mandates caution when choosing the appropriate MN assay for measuring DNA damage and in the interpretation of results (Table II and Figure 1). Absence of splenic function and high reticulocyte percentage in SCA suggests the erythrocyte MN assay without enrichment for CD71+ cells is suitable for this disorder. In contrast, severe thalassaemia is characterised by low reticulocyte number and presence of transfused erythrocytes from healthy donors. Hence, the erythrocyte MN assay is inappropriate for thalassaemia, even in splenectomised patients. Under these conditions, enrichment of whole blood for Trf–reticulocytes is required to measure endogenous MN-frequency (Figure 1b and e).

Recommendations for future research

Validity of MN measurements in blood cells to the other tissues in the body

As a result of the continuous DNA synthesis in the bone marrow to replenish blood cells, the haematological system demonstrates exquisite sensitivity to DNA-damaging agents (61,62). Direct DNA toxins such as cancer chemotherapy agents and ionising radiation produce their earliest and maximum effect on the bone marrow (63). Coexisting micronutrient deficiencies may enhance the effect of DNA-damaging agents on blood cells. While this indicates that evaluation of DNA damage in blood cells is a sensitive test for micronutrient deficiencies (28), the extrapolation of MN-frequency in reticulocytes or lymphocytes to the rest of the tissues in the body deserves further reflection. The relevance of this issue is demonstrated by the fact that the majority of cancers in the elderly arise from solid organs and not blood cells (64). At the current time, corroborative studies between the erythrocyte or lymphocyte MN assay and DNA damage occurring in other tissues are rare (13), but the MN frequency in lymphocytes may be higher than in exfoliated buccal cells (65). Increased use of exfoliated cells from buccal, respiratory, genitourinary or gastrointestinal tract may prove useful to study the effect of genotoxicity in other organ systems (13,66).

Table II. Comparison of haematological values

	Normal	SCA	Thalassaemia	Hereditary spherocytosis
Haemoglobin	N	↓↓	↓↓↓	↓
Reticulocytes	N	↑↑↑	↑	↑↑
Ineffective erythropoiesis	N	↑	↑↑↑	N
Splenic function	N	Absent	N ^a	N ^a
Oxidative stress	N	↑	↑↑↑	N
Nutritional deficiencies	N ^b	↑↑↑	↑↑↑	↑

N, normal.
^aMany individuals are splenectomised.
^bFollow population levels.

Significance of elevated erythrocyte MN frequency in haematological diseases

Haematological diseases, particularly the chronic haemolytic anaemias, are an attractive model to study effect of micronutrient deficiencies owing to high reticulocyte counts and absence of splenic function. However, the pathophysiological process in these disorders can interfere with cell division and induce cell death or abnormal nuclear maturation (67). These disease-specific events involve mechanisms such as precipitation of proteins, catalytic iron and apoptosis (52,67), which are largely independent of the micronutrient status. Hence, much further experimental work remains to be done to understand the implications of elevated erythrocyte MN frequency documented in thalassaemia and SCA (38,40). The corollary is that some haemolytic anaemias, in which the pathophysiological process targets the mature RBC, but the erythroid development remains unaffected, are superior models to study the effect of micronutrient deficiency or genotoxic stress. Hereditary spherocytosis and some erythrocyte enzyme deficiencies are examples of such disorders (68). Many patients with hereditary spherocytosis have undergone splenectomy, which would make them suitable candidates to easily follow the rate of erythrocyte MN formation. Comparison of patients with hereditary spherocytosis with SCA or thalassaemia will provide much needed assessment of the impact of pathological erythroid development on MN formation in erythrocytes.

Micronutrient deficits and oxidative stress in haematological diseases and MN formation

Chronic haemolytic anaemias, thalassaemias and SCA, are associated with significant increase in oxidant production and depletion of endogenous antioxidants and several micronutrients (46,55). Future studies are needed to understand the role of antioxidant therapy to reverse oxidative stress for the prevention of DNA damage in these diseases. Research is also needed to evaluate sufficiency of several micronutrients, such as vitamin C, vitamin E, selenium and zinc, in haematological diseases whose requirement may be higher than in normal population.

Funding

National Center for Complementary and Alternative Medicine (R21AT004493); Bruce and Giovanna Ames Foundation.

Acknowledgements

We are also indebted to Joyce McCann for her helpful criticisms of this manuscript. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center for Complementary & Alternative Medicine or the National Institutes of Health.

Conflict of interest statement: None declared.

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